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Selection of plants for roles in phytoremediation: the importance of glucosylation

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Summary

Over-expression and transposon mutagenesis in root cultures of *Arabidopsis thaliana* demonstrated the importance of the family 1 glycosyltransferase UGT72B1 in catalysing the *N*-glucosylation of the persistent pollutant 3,4-dichloroaniline (DCA). In phytotoxicity studies with DCA in seedlings, over-expression of UGT72B1 enhanced sensitivity, whereas the knockouts were more resistant than the controls. In contrast, manipulating the expression of UGT72B1 had no effect on the *O*-glucosylation, or toxicity, of chlorophenols. When *N*-glucosylation was disrupted in plants, radioactivity derived from [^{14}C]-DCA became covalently bound into high molecular weight insoluble material, principally associated with the lignin fraction. This suggested that insolubilization into stable cell wall components represented a more effective mechanism of DCA detoxification than the formation of *N*-glycosidic conjugates. A screen of plants used in remediation, identified low levels of *N*-glucosyltransferase activity in switchgrass and high activities in reed canary grass. When incubated with [^{14}C]-DCA, reed canary grass plants accumulated soluble *N*-glycosides of DCA, whereas switchgrass formed insoluble residues. Consistent with the results obtained in studies with *Arabidopsis*, phytotoxicity trials with DCA demonstrated that switchgrass was more tolerant than reed canary grass. Our studies provide a new biochemical basis for selecting plants for useful remediating traits towards specific classes of pollutants.

Keywords: bound residues, chloroanilines, lignin, *N*-glucosylation, phytotoxicity.

Introduction

The use of plants to phytoremediate environments contaminated with organic pollutants has attracted considerable interest as representing a low-cost and sustainable solution to environmental improvement, as compared with costly intervention using chemical or engineering approaches (Meagher, 2000). As part of a long-term programme we have been interested in identifying useful proteins in plants which confer tolerance to synthetic organic compounds, including pollutants, which could then be harnessed in biotechnological applications such as bioremediation. One group of proteins that can be readily identified as being potentially useful biocatalysts for detoxifying xenobiotics are the family 1 glycosyltransferases (UGT). These enzymes catalyse the *O*-, *S*- and *N*-glyco-conjugation of a diverse range of synthetic compound acceptors using NDP-activated sugar donors (Schröder *et al*., 2001). As such, they are a very important group of bioconjugating, or phase 2, enzymes that are involved in the detoxification of multiple pollutants and pesticide metabolites in plants (Cole and Edwards, 2000).

In recent studies we identified a UGT in *Arabidopsis thaliana*, termed UGT72B1, which catalysed the conjugation of both 3,4-dichloroaniline (DCA) and 2,4,5-trichlorophenol (TCP) with glucose (Loutre *et al*., 2003). Both DCA and TCP are persistent pollutants which are widespread in environments exposed to effluents from chemical industries, or heavy pesticide usage. In the European Union both compounds are designated priority pollutants (Harvey *et al*., 2002). When UGT72B1 was selectively disrupted by transposon mutagenesis, the resulting *ugt72B1* knockout *Arabidopsis* plants were impaired in their ability to glucosylate DCA, but were unaffected in their conjugation of TCP (Brazier-Hicks and Edwards, 2005). Unexpectedly, *ugt72B1* seedlings showed an increased tolerance to DCA as compared with wild-types, prompting us to further investigate the role of UGT72B1 in

xenobiotic detoxification *in planta*, by generating and testing the respective over-expressors (UGT72B1-OEs). Using the results obtained from metabolism and toxicity studies in these *Arabidopsis* lines, we have then used this biochemical information to select for plant species able to tolerate and remediate DCA.

Results

Effect of manipulating the expression of UGT72B1 on the metabolism of xenobiotics in *Arabidopsis*

To investigate the effects of over-expression of UGT72B1 on the metabolism of xenobiotics, a UGT72B1-pCAMBIA 3300 construct was created and transformed into *Arabidopsis* (Dixon *et al.*, 2003). Seed from homozygous T3 lines were used to create root cultures for metabolism and protein expression studies. Root cultures were used, as UGT72B1 expression could be accurately monitored by Western blotting without interference from comigrating Rubisco protein (Brazier-Hicks and Edwards, 2005). In addition, the use of root cultures facilitated the quantitative monitoring of the distribution of the radioactivity between the plant and root medium and helped reduce the large-scale loss of volatile radioactivity seen with [^{14}C]-DCA in whole plant studies (Brazier-Hicks and Edwards, 2005). Levels of immunoreactive polypeptides in the T3 UGT72B1-pCAMBIA transformant lines were determined by immunoblotting using a specific anti-UGT72B1-serum (Brazier-Hicks and Edwards, 2005). Two lines showing enhanced expression of UGT72B1 were identified, with one line selected for further characterization and designated UGT72B1-OE. Enhanced levels of the UGT72B1 polypeptide were determined in UGT72B1-OE roots as compared to the wild-type (Figure 1a). However, as determined by Western blotting, the level of immunoreactive protein in the *ugt72B1* roots was below the limit of detection (2 ng) as determined with recombinant UGT72B1. The extracts were assayed for *O*-glucosyltransferase (OGT) conjugating activity towards chlorophenols and *N*-glucosyltransferase (NGT) activity towards chloroaniline substrates using UDP-[^{14}C-glucose] as the sugar donor (Table 1). As compared with control plants, knocking out the expression of UGT72B1 reduced extractable enzyme activity towards DCA (101-fold) and 2,4,5-trichloroaniline (11-fold), while with the chlorophenols, 27% and 63% of OGT activity was retained with the substrates TCP and triclosan, respectively (Table 1). Over-expression of UGT72B1 gave a four- to sevenfold enhancement in NGT activity and a two- to fourfold increase in OGT, depending on the substrate assayed.

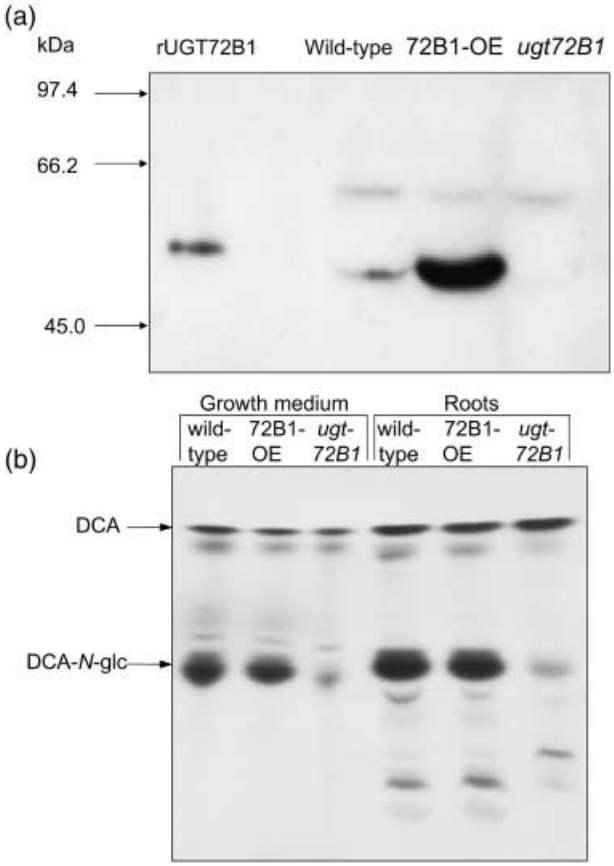
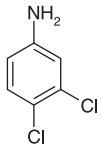
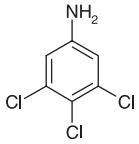
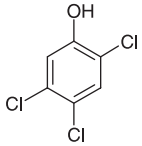
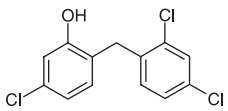


Figure 1 The effect of manipulating UGT72B1 expression in *Arabidopsis* root cultures, by gene knockout (*ugt72b1*) or over-expression (UGT72B1-OE) as compared with wild-types. (a) Western blot analysis of crude protein extracts (100 μg) following resolution by SDS-PAGE and probing with an anti-UGT72B1-serum. For reference, recombinant UGT72B1 was also analysed, with the positions of the molecular mass markers arrowed. (b) Autoradiographs of the radioactive metabolites resolved by thin layer chromatography formed in the three lines after treating with [^{14}C]-DCA for 24 h. Parent DCA and the major metabolite DCA-*N*-glucoside are indicated.

To determine the effect of perturbing UGT72B1 expression on the conjugation of chlorinated xenobiotics *in planta*, radiolabelled DCA and TCP were individually fed to wild-type, UGT72B1-OE and *ugt72b1* root cultures. As described previously, knocking out the expression of UGT72B1 had no significant effect on uptake of the radioactivity following feeding with [^{14}C]-TCP (Brazier-Hicks and Edwards, 2005). Thus, over an 8-h incubation, 54.3% ± 6.9% (mean ± variation, $n = 2$) of the recovered radioactivity was present in the roots of the wild-types, as compared with a figure of 59.5% ± 8.3% in the knockouts. Similarly increasing the expression of the enzyme had no detectable effect on the quantitative partitioning of the radioactivity, with 59.2% ± 2.3% recovered in the roots. In each of these studies with [^{14}C]-TCP the overall recoveries of radioactivity were similar, being in the range

Table 1 Glucosyltransferase (GT) activity towards chloroanilines and chlorophenols in protein extracts from *Arabidopsis* root cultures where UGT72B1 has either been over-expressed (UGT72B1-OE) or knocked out (*ugt72B1*). Activity in wild-type cultures are shown for comparison. Values are means ± SD (n = 3)

Substrates		GT activity toward xenobiotics (pmol/min/mg protein)		
		Wild-type	UGT72B1-OE	*ugt72B1*
NGT				
3,4-Dichloroaniline (DCA)		101.1 ± 14.9	459.8 ± 27.7	1.0 ± 0.2
3,4,5-Trichloroaniline		3.3 ± 0.0	22.4 ± 2.8	0.3 ± 0.0
OGT				
2,4,5-Trichlorophenol (TCP)		32.8 ± 6.2	129.7 ± 0.8	9.1 ± 1.3
Triclosan		14.7 ± 0.0	34.1 ± 5.9	9.4 ± 1.0

70.2 ± 6.6% of the applied dose. Similar losses in recovered radioactivity were observed in earlier metabolism studies (Brazier-Hicks and Edwards, 2005), although it seems unlikely that these are due to direct volatilization of TCP (boiling point, 253 °C). Quantification of the radioactive metabolites by thin layer chromatography (TLC) showed that the *O*-glucoside of [^{14}C]-TCP was the major extractable metabolite in both the roots and the medium of all three lines representing just under 50% (49.1% ± 5.6%) of the total recovered radioactivity in each case after an 8-h incubation. It therefore appeared that *Arabidopsis* OGTs, other than UGT72B1, with activity towards TCP masked the effect of varying UGT72B1 expression on the rates of *O*-conjugation of this chlorophenol in the root cultures. Thus, several *Arabidopsis* UGTs other than UGT72B1 are known to actively glucosylate TCP (Messner *et al*., 2003).

With [^{14}C]-DCA, the effect of knocking-out UGT72B1 was to reduce the initial rate of uptake of the xenobiotic from the media into the roots, suppress the levels of soluble radioactivity accumulating in the plant tissues and greatly enhance the formation of non-extractable insoluble radioactive residues (Figure 2). Surprisingly, over-expression of UGT72B1 had a negligible effect on the overall distribution of soluble radioactivity accumulating in the root tissues and in the media, although as compared with wild-type cultures, a reduction in insoluble residue formation was determined at 24 h (Figure 2). Total recoveries of radioactivity over the period of the experiment were 67.4% ± 3.7% (mean ± variation, n = 2) of the applied dose in the wild-type cultures, 64.3% ± 2.1% for the over-expressors, and 54.2% ± 2.7% for the knockouts. Figures for recoveries were similar to those determined in earlier studies and were due to the volatilization of [^{14}C]-DCA from the media, with this being greater in the *ugt72b1* cultures (Brazier-Hicks and Edwards, 2005). To monitor the effect of varying UGT72B1 expression on the formation of specific DCA metabolites, the methanol-extractable radioactive residues in the roots and medium from knockout, wild-type and over-expressing lines were analysed by TLC and autoradiography (Figure 1b). The major radioactive metabolites in both the medium and the roots were parent DCA and DCA-*N*-glucoside, respectively, with the identity of these metabolites confirmed by HPLC-MS (Loutre *et al*., 2003). The more minor polar metabolites were additional uncharacterized *N*-glycosidic conjugates (Loutre *et al*., 2003). The results demonstrated that while the *ugt72B1* plants were impaired in their ability to conjugate DCA, over-expression of UGT72B1 had a negligible effect on *N*-glucosylation of the xenobiotic.

The metabolism studies demonstrated that UGT72B1 played an important role in determining whether or not radioactivity

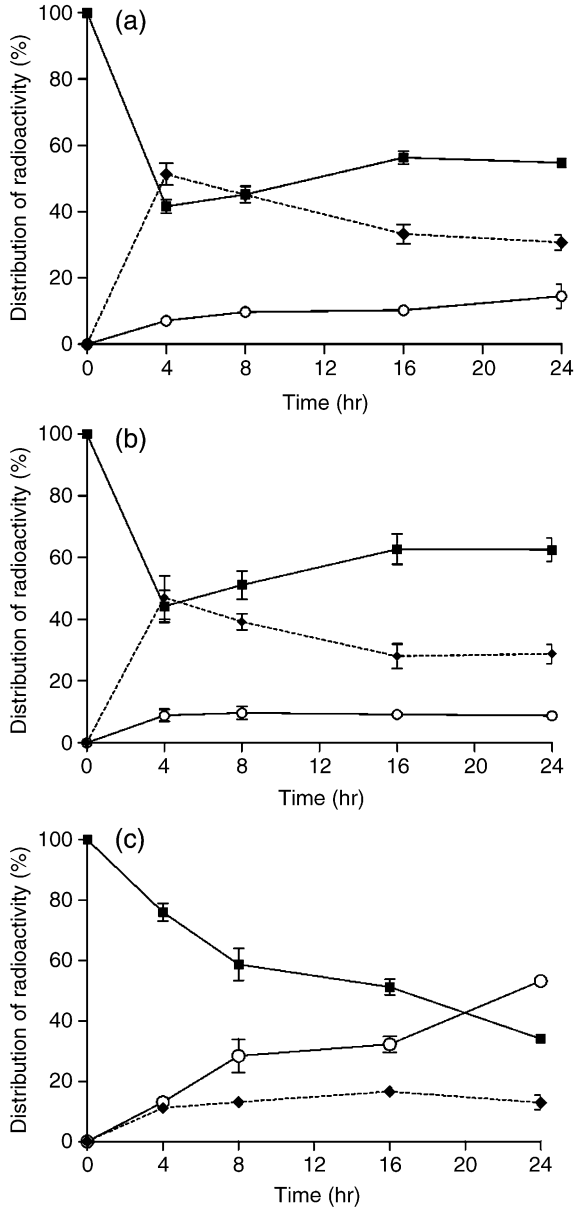


Figure 2 The distribution of radioactivity (% recovered dose) in the medium (■), solvent-extractable (◆) and nonextractable (○) fractions from root tissue following the feeding of 100 nmol of [^{14}C]-DCA to (a) wild-type, (b) UGT72B1-OE and (c) *ugt72B1 Arabidopsis* cultures. Results represent the means of duplicate determinations with error bars showing the variation in the means. Dosing, harvesting and analysis were as described (Brazier-Hicks and Edwards, 2005).

derived from [^{14}C]-DCA became incorporated into the insoluble, or 'bound' residues. To investigate the nature of the bound residues, wild-type and knockout cultures were treated with [^{14}C]-DCA for 24 h to promote incorporation. Following solvent extraction, the insoluble residue was then sequentially extracted with chemical and enzymic treatments to fractionate the insoluble radioactive residues as being associated with

Table 2 Radioactive residues recovered in the soluble and insoluble fractions of wild-type and *ugt72B1 Arabidopsis* root cultures dosed with 240 nmol of [^{14}C]-DCA for 24 h. Values represent means ± SD (n = 3)

		Extracted [^{14}C]DCA (nmol)	
Fraction		Wild-type	*ugt72B1*
Soluble		87.59 ± 5.00	90.72 ± 5.00
Insoluble			
Ionically bound	1	1.50 ± 0.33	4.05 ± 0.30
Starch	2	0.68 ± 0.05	2.25 ± 0.17
Protein	3	1.73 ± 0.23	4.93 ± 0.26
Pectin	4	3.05 ± 0.13	12.48 ± 0.47
Lignin	5	10.47 ± 1.70	46.00 ± 4.44
Hemicellulose	6	1.85 ± 0.31	12.63 ± 3.23
Cellulose	7	0.53 ± 0.22	2.48 ± 1.31
Total		19.81	84.82

definable bio-macromolecules (Langbartels and Harms, 1985). As compared with the wild-types, the *ugt72b1* plants contained higher levels of solubilized radioactivity in all of the fractions derived from the methanol-insoluble residue (Table 2). In both sets of samples, the single greatest recovery of radioactivity was obtained following extraction with dioxane-based solvents, corresponding to the selective solubilization of the lignin fraction (Langbartels and Harms, 1985). In the knockout plants, approximately 25% of the total radioactivity extracted from the plant material was present in the lignin fraction, as compared with a figure of around 10% in the controls.

Role of UGT72B1 in determining the toxicity of chlorinated anilines and phenols to *Arabidopsis* plants

The metabolism and enzyme assays all pointed to an important role for UGT72B1 in regulating the metabolism of chloroanilines. To determine the effect of manipulating UGT72B1 expression on the phytotoxicity of DCA, seedlings from the wild-type, *ugt72b1* and UGT72B1-OE lines of *Arabidopsis* were germinated on agar containing increasing concentrations of the pollutant (Figure 3). As reported previously (Loutre *et al*., 2003), the *ugt72B1* seedlings were found to tolerate concentrations of DCA greater than 25 μM much better than the wild-type. In contrast, the over-expressors showing an enhanced sensitivity to DCA at concentrations above 10 μM. In subsequent studies, the toxicity trials were extended to include 2,4-dichloroaniline, 3,4,5-trichloroaniline, 2,4,5-trichlorophenol, 2,3,4-trichlorophenol, 2,3,6-trichlorophenol and triclosan (Figure S1). With the two chloroanilines, the results mirrored those observed with DCA, with reduced expression

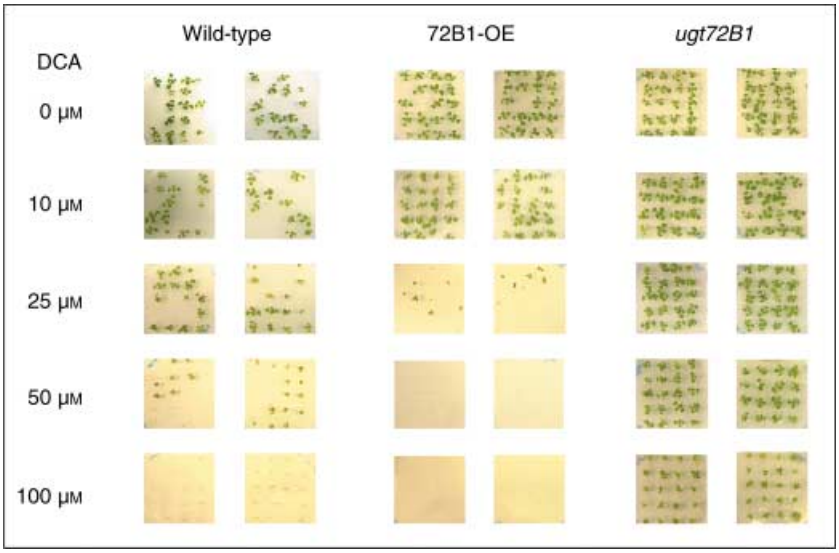


Figure 3 Phytotoxicity of DCA in *Arabidopsis* seedlings showing modified expression of UGT72B1. Seeds of wild-type, knock-out (*ugt72b1*) and over-expressing (UGT72B1-OE) lines were germinated on agar containing 0–100 μM DCA. Scoring data and additional phytotoxicity trials with other xenobiotics are given in the supplementary data.

of UGT72B1 reducing toxicity and increased expression enhancing sensitivity. With the chlorophenols, no significant effect on tolerance was determined after varying UGT72B1 expression through either knockout or over-expression (Figure S1).

Screening plants for useful traits in DCA phytoremediation based on relative NGT activity

Our results with *Arabidopsis* demonstrated that the titres of NGT conjugating activity towards DCA determined tolerance towards the chloroaniline. Since NGTs active in DCA conjugation are not unique to *Arabidopsis* (Pflugmacher and Sandermann, 1998), plants with potential utility in phytoremediation were screened for NGT activity. The first plants selected were *Brassica napus*, *Brassica juncea* and *Brassica nigra*, which have proven useful in remediation studies with inorganic and organic pollutants in both laboratory and field studies (Marchiol *et al*., 2004). Conveniently, these *Brassica* species are also closely related to *Arabidopsis*. In addition, switchgrass (SWG) and reed canary grass (RCG) were selected as two fast-growing grasses with similar growth habits, which are finding increasing applications both in remediation and as biofuel crops. For example, RCG and SWG have been used in field trials to remediate explosives and polyaromatic hydrocarbons, respectively (reviewed by Singh and Jain, 2003). The three *Brassica* species all contained very low NGT activity towards DCA, while conjugating activity towards TCP was determined in the range 5.6–10.3 pmol/min/mg protein (Table 3), which was similar to that determined for *Arabidopsis* plants (8.5 pmol/min/mg). Since the *Brassica* species had such similar GT activities to one another, they were unsuitable for comparing the role of metabolism in determining the toxicity of DCA, or indeed TCP. Also because the size of the plants was so different from *Arabidopsis*, comparative metabolism and phytotoxicity studies between the *Brassica* and the model species would have been of little quantitative value. Instead, while the two cultivars of RCG showed high NGT activity, both SWG lines showed very low activities towards DCA (Table 3). All of the grasses had similar OGT activities towards TCP. Thus, these two grass species offered a better system to compare the roles of NGTs in determining the routes and rates of metabolism and subsequent sensitivity to chloroanilines.

Table 3 Glucosyltransferase (GT) activities towards xenobiotics in crude protein extracts from the foliage of a range of plant species. Values are the means ± SD (n = 3)

	GT activity (pmol/min/mg crude protein)	
Plant species (cultivar)	3,4-Dichloroaniline	2,4,5-Trichlorophenol
Arabidopsis	14.9 ± 0.8	8.5 ± 0.7
Brassica napus	0.2 ± 0.0	5.6 ± 0.8
Brassica juncea	0.5 ± 0.0	8.3 ± 0.4
Brassica nigra	1.0 ± 0.4	10.3 ± 0.5
Reed canary grass 'Bamse'	6.1 ± 0.7	2.1 ± 0.2
Reed canary grass 'Palaton'	6.3 ± 0.2	1.8 ± 0.0
Switchgrass 'Shelter'	0.2 ± 0.0	1.3 ± 0.0
Switchgrass 'Trailblazer'	0.5 ± 0.4	2.3 ± 0.4

RCG and SWG were incubated with [^{14}C]-DCA for 24 h and the methanolic extracts then analysed by TLC (Figure S2). Both the Palaton and Bamse cultivars of RCG formed DCA-*N*-glucoside as a major metabolite. Two more polar metabolites were also observed, which based

Table 4 Uptake of [^{14}C]-DCA in reed canary grass and switchgrass over a 24-h exposure, with the distribution of radioactivity between the medium and bound and extractable fractions from the grasses shown. The nature of the soluble radioactive metabolites is shown in Fig. S2

	Radioactivity derived from [^{14}C]-DCA (nmol)			
	Reed canary grass		Switchgrass	
	Palaton	Bamse	Trailblazer	Shelter
Medium	24.7 ± 1.8	24.6 ± 0.9	24.7 ± 1.0	23.6 ± 1.5
Plant extract	4.6 ± 1.1	3.9 ± 0.0	1.7 ± 0.2	2.4 ± 0.9
Plant non-extract	0.3 ± 0.1	0.2 ± 0.0	3.1 ± 0.2	3.9 ± 0.4
Total	29.6	28.7	29.5	29.9

Table 5 Phytotoxicity of DCA in reed canary grass and switchgrass. The effect of different concentrations (0–100 μM) of DCA on plant height determined as mean values ± SD (n = 6). Photographs showing the effect of 100 μM DCA on the grasses is shown in Fig. S3

	Phytoxicity study (plant height in cm)			
	Reed canary grass		Switchgrass	
	Palaton	Bamse	Trailblazer	Shelter
Control	26.5 ± 2.0	30.8 ± 2.6	24.5 ± 0.2	24.5 ± 1.3
10 μM DCA	29.3 ± 2.1*	30.0 ± 5.5*	26.2 ± 2.8*	25.0 ± 1.7*
50 μM DCA	20.5 ± 2.1‡	18.7 ± 2.1‡	21.1 ± 1.7‡	22.0 ± 1.9†
100 μM DCA	16.3 ± 3.5‡	13.2 ± 1.4‡	20.7 ± 2.0‡	19.0 ± 0.6‡

*no significant difference ($P > 0.1$).

†$P < 0.01$; ‡$P < 0.001$ in comparison with control values in each case.

on their co-chromatography with DCA metabolites isolated from *Arabidopsis*, were uncharacterized *N*-glycosidic conjugates (Loutre *et al*., 2003). In the two SWG lines, DCA-*N*-glucoside and one of the polar *N*-glycosides was also observed at lower abundance, with the major extractable metabolite residing near the origin. The radioactivity in the extractable and non-extractable fractions of the grasses fed with [^{14}C]-DCA was then determined (Table 4). The uptake of radioactivity in the RCG and SWG plants was nearly identical, with the majority of administered [^{14}C]-DCA remaining in the medium. Total recoveries of radioactivity in the experiments with SWG and RCG were very similar (69 ± 4%), with the missing radioactivity due to the volatilization of [^{14}C]-DCA, as determined with *Arabidopsis* plants previously (Brazier-Hicks and Edwards, 2005). In the RCG plants the majority of the absorbed radioactivity was recovered in the extractable fraction, while in the SWG plants bound residues predominated. Interestingly, parent [^{14}C]-DCA was easily detected in the extractable fraction from the RCG plants but not in SWG, which was surprising based on the lower NGT activities in the latter species. From this it was conjectured that in the SWG plants, the DCA was being removed from the extractable fraction more effectively than was the case in RCG. This corresponded to the appearance of the very polar radioactive metabolite on the autoradiogram (Figure S2), which was possibly an intermediate of bound residue formation.

To determine whether or not the differences in the routes of DCA metabolism in RCG and SWG were reflected in differences in tolerance to the pollutant, seedlings from the two species were treated with increasing concentrations of DCA and the effect on plant growth determined (Table 5 and Figure S3). In RCG, concentrations of DCA above 50 μM inhibited growth, such that at 100 μM the plants were only half the height of the untreated controls at the conclusion of the study. In the SWG plants a negligible inhibition of growth was determined at 50 μM while at 100 μM, final plant height was suppressed by only 20% (Table 5).

Discussion

Chloroanilines such as DCA are common environmental pollutants and pesticide metabolites and are known to undergo three major alternative routes of metabolism in higher plants, namely, *N*-glucosylation, *N*-malonylation or bound residue formation (Schmidt *et al*., 1994). A schematic diagram showing these three reactions is shown in Figure 4. In the case of the insoluble plant residues, DCA is known to be incorporated into several plant macromolecules, predominantly lignin (reviewed by Sandermann, 2004). As observed in our fractionation studies, incorporation of chloroanilines occurs in the early stages of polymerization, with reactions with phenoxy radicals leading to the DCA substituting at the benzylic α-position of the lignin side chains (Lange *et al*., 1998), as shown in Figure 4. In *Arabidopsis*, where *N*-glucosylation is the major route of DCA metabolism (Lao *et al*., 2003), we have identified competing pathways of sugar conjugation and bound residue formation. Thus, when NGT activity is suppressed *in planta*, DCA is directed into the lignin fraction. This competition model for DCA also seems to operate in the grasses RCG and SWG. In the RCG plants, which contained high levels of NGT activity, DCA was metabolized to the *N*-glucoside, whereas in SWG, which had low levels of this conjugating activity, bound residues predominated. Insoluble residue formation has also been reported in plants that

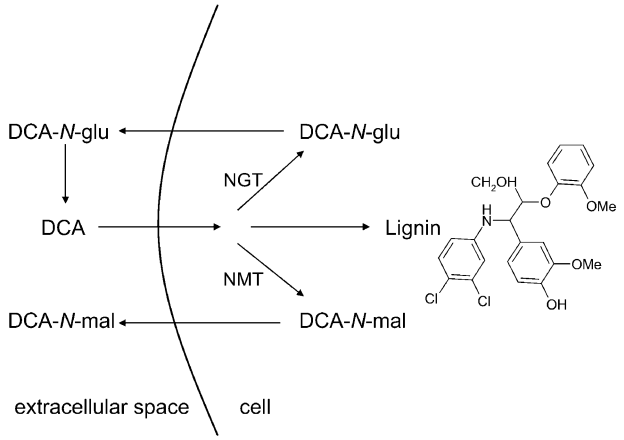


Figure 4 The metabolism and disposition of DCA in plants, showing the potential roles of the *N*-malonyltransferase (NMT) and *N*-glucosyltransferase (NGT) in forming the respective DCA-*N*-malonate and DCA-*N*-glucoside. The bound residue shown is a lignan dimer with the DCA substituted at the α-benzylic as described previously (Lange *et al*., 1998).

use a combination of *N*-glucosylation and *N*-malonylation to metabolize DCA, such as soybean, wheat and carrot (Bockers *et al*., 1994; Schmidt *et al*., 1994). As shown in Figure 4, in such plants *N*-malonylation will also compete for DCA with the *N*-glucosylation and bound residue pathways (Winkler and Sandermann, 1989; Gareis *et al*., 1992). Conjugation by *N*-glucosylation and *N*-malonylation both direct DCA for extracellular deposition (Gareis *et al*., 1992; Lao *et al*., 2003). Once exported, these conjugates are only poorly reabsorbed (Lao *et al*., 2003). However, although the DCA-*N*-malonate is described as being stable to chemical and microbial hydrolysis (Winkler and Sandermann, 1989), this is not the case with DCA-*N*-glucoside (Winkler and Sandermann, 1992). Thus, following its export into the rhizosphere, the glucoside will undergo chemical and microbial hydrolysis to release DCA, which will then be re-imported into the plant via the roots (Figure 4). In plants that predominantly metabolize DCA by *N*-glucosylation, this cyclical re-absorption of the pollutant means that its incorporation into the cell wall is the most effective mechanism of sequestration. Once bound, the residues have the potential to be completely removed from the environment if the plant is harvested, or will return to the soil as partly degraded lignin derivatives once the dead tissue is acted on by microbes (Harvey *et al*., 2002; Sandermann, 2004). In the current report, based on the assumption that all plants have the capacity to peroxidatively cross-link DCA into cell wall components, we have only considered low glucosylating activities as a metabolic trait likely to predispose plants to hyperaccumulate bound residues. This is likely to be a reasonable model based on the widespread occurrence of NGT activity towards DCA in higher plants (Pflugmacher and Sandermann, 1998). However, a further useful refinement for a predictive screen for bound residue formation in different species would be the determination of the less widely reported DCA-*N*-malonyltransferase activity (Lao *et al*., 2003).

By disrupting the expression of UGT72B1 in *Arabidopsis*, our studies have demonstrated the practical importance of the competition between soluble and insoluble pathways of DCA detoxification in determining tolerance to this pollutant. We have then shown that the inverse relationship between the rates of *N*-glucosylation and phytotoxicity of DCA also appear to operate in two grass species. While additional factors may also regulate the toxicity of DCA in the grasses, the feeding studies demonstrated that the chloroaniline was taken up with similar efficiency in all the cultivars of RCG and SWG tested, demonstrating that differences in tolerance were not due to variations in the bioavailability of the pollutant. Although we cannot demonstrate that the grasses fundamentally differ in their sensitivity to DCA, our results are consistent with SWG being more tolerant than RCG to DCA because of its greater capacity to incorporate the pollutant into a stable insoluble residue.

The results of our study are important for plant biotechnology for two reasons. First, they provide a biochemical basis for deploying plants in specific applications for the removal of specific chloroaniline pollutants from the environment. While DCA is only classified as a priority pollutant in the European Union, related chloroanilines are commonly determined pollutants worldwide, being degradation products of pesticides and intermediates in the synthesis of azo dyes. As long-lived contaminants, they are particularly toxic to aquatic organisms and the utility of plants expressing low levels of NGTs to scavenge these compounds from the environment to form stable bound residues has a number of attractions (Harvey *et al*., 2002). Since many pollutants have the potential to undergo metabolism to both soluble and bound residues (Sandermann, 2004), a greater knowledge of how these pathways compete for detoxification *in planta* could be extremely useful in selecting plants for the remediation of specific pollutants, especially if these traits can be harnessed in emerging bioenergy crops. In addition to providing new tools for phytoremediation, our studies also give new insight into the factors regulating bound residue formation in plants. Thus, understanding the competition between routes of soluble and insoluble residue formation is an important biotechnological tool in predicting the metabolism of crop protection agents and the form in which xenobiotics enter the food chain (Sandermann, 2004).

Experimental procedures

Plants

Plants and root cultures of wild-type and transgenic *Arabidopsis thaliana* (Columbia) were maintained as described previously (Brazier-Hicks and Edwards, 2005). Seeds of *B. napus*, *B. juncea* and *B. nigra* were obtained from Herbiseed, Wokingham, UK, and of RCG (*Phalaris arundinacea* vars. Palaton and Bamse) and SWG (*Panicum virgatum* vars Shelter and Trailblazer) from Semundo Ltd. (Great Abington, Cambridge). For the metabolism studies, *Brassica* plants and the grasses were placed on moist tissue paper for 3 days in the light and then grown in Arthur Bower's multipurpose compost for 21 days. *Arabidopsis* plants over-expressing UGT72B1 were generated by subcloning UGT72B1 from pET11d into the expression vector cassette pRT108 using *Nco*I and *Bam*HI sites. This was then digested with *HindIII* and ligated into the binary vector pCAMBIA 3300 (CAMBIA, Canberra, Australia). The vector was used to transform *Arabidopsis* plants (Col-0) and the resulting seed subjected to selection with glufosinate ammonium using a previously described procedure (Dixon *et al*., 2003). Selection was repeated through a further two generations to produce the homozygous T3 UGT72B1-OE transformed plants. The T-DNA knockout line *ugt72B1* was available from previous studies (Brazier-Hicks and Edwards, 2005).

Feeding studies with radiolabelled xenobiotics

For metabolism studies *Arabidopsis* root cultures (14 days old) were dosed for 24 h with 4 μM [U-^{14}C]-TCP (74 MBq/mmol, Sigma, Poole, Dorset, UK) or [U-^{14}C]-DCA (769.6 MBq/mmol, Sigma), the latter diluted with cold DCA to a final specific activity of 74 MBq/mmol. Cultures were harvested at 4 h, 8 h, 16 h and 24 h and the roots and media separated and extracted as described previously (Brazier-Hicks and Edwards, 2005). Extracted radioactive residues were quantified by liquid scintillation counting (LSC) prior to TLC using the solvent system chloroform : methanol : water (60 : 35 : 8 v/v). Radioactive metabolites were located by autoradiography and quantified by LSC. Where possible, identification was based on co-chromatography with reference metabolites that had previously been identified by mass spectrometry (Loutre *et al*., 2003). The total bound residue remaining after extraction with methanol was combusted using a sample oxidizer and the $^{14}CO_2$ released captured and quantified by LSC (Brazier-Hicks and Edwards, 2005).

For metabolism studies with RCG and SWG, the stems of 14-day-old plants were placed in 4 μM [^{14}C]-DCA prepared at the same specific activity as described for the studies with *Arabidopsis* root cultures. After 24 h, the plant tissue and feeding solution were extracted and analysed by LSC and TLC.

Fractionation of bound residues

The method for sequential fractionation of bound residues was adapted from a published procedure (Langbartels and Harms, 1985). Plant tissue (0.5 g, $n = 4$) was extracted with methanol (10 mL) for 4 h at 4 °C on an end-over-end mixer after homogenizing with a pestle and mortar. Solvent extraction was repeated a further four times, until no further methanol-soluble radioactivity was released. The residue was then washed with water, followed by 0.1 M K phosphate buffer pH 7.0 containing 0.15 M NaCl (4 h) to release ionically bound residues. The insoluble material was then sequentially extracted with the following treatments (each 10 mL unless specified otherwise) under the conditions given in parentheses. Amylase (2% v/v from *Bacillus* sp. Novozyme) in 0.1 M K phosphate buffer pH 7.0 (16 h, 37 °C) was used to hydrolyse starches; pronase (1 mg/mL from *Streptomyces griseus*, Fluka) in 0.1 M Tris-HCl pH 7.2 (8 h, 37 °C) was used to digest protein; 50 mM EGTA in 50 mM sodium acetate pH 4.5 (16 h, 80 °C) was used for pectin solubilization; dioxane : water (9 : 1 v/v, 16 h, 80 °C) followed by dioxane : aqueous 2 M HCl (9 : 1 v/v, 8 h, 80 °C) were used in sequence and then pooled to derive a soluble lignin extract: 4.3 M KOH was employed to digest hemicelluloses (4 h, 80 °C). The final residue was treated with concentrated H_2SO_4 to hydrolyse cellulose (1 mL, 25 °C, 4 h) and then diluted with water (9 mL) prior to LSC. Following each treatment, residual solid material were recovered by centrifugation (3900 *g*, 5 min) and resuspended by ultasonication prior to digestion. Extracts (100 μL) from each extraction were radioassayed in duplicate by LSC, with the hemicellulose digest neutralized with acetic acid prior to analysis.

Phytotoxicity trials

For studies with *Arabidopsis*, each replicate ($n = 3$) consisted of a Petri dish sown with 60 seeds equally spaced out on to MS agar containing a range of concentrations (0–100 μM) of chlorinated anilines or phenols. Seedlings grown on plates containing agar alone were used as untreated controls. After 14 days, plates were scored for toxicity as follows: score of 10 = full germination and growth as observed in controls; score of 9 to 3 = 90% to 30% of growth observed in controls; score of 2 = cotyledons expanded but undergoing necrosis; score of 1 = germination halted following initial root emergence; and a score of 0 = no germination.

For studies with RCG and SWG, seedlings were germinated on blotting paper and after 7 days carefully transplanted into perlite, Plants were maintained in a growth room using the conditions described above, with Phostrogen used for nutrient. After 20 days, plants were treated with DCA dissolved in water (0–100 μM) and scored for phytotoxicity after 12 days of treatment by determining plant height (Clark *et al*., 2004).

Enzyme analysis

Glucosyltransferase activity in crude plant extracts was determined by radioassay using UDP-[^{14}C-glucose] as described (Loutre *et al*., 2003). The presence of immunodetectable UGT72B1 was determined by Western blotting using an antiserum raised against the respective recombinant protein (Brazier-Hicks and Edwards, 2005).

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Effect of modified UGT72B1 expression on the tolerance of *Arabidopsis* seedlings to chlorinated phenols and anilines. Plants used were derived from wild-type, over-expressor (72B1-OE) and knockout (*ugt72b1*) lines. (A) Images of plants showing a score of 10 = full germination and maximum growth (i.e. untreated plants), score of 4 = 40% of growth observed in untreated controls, score of 2 = cotyledons expanded but undergoing necrosis, score of 1 = germination halted at root elongation and score of 0 = no germination. (B) Growth/toxicity scores of 14 day old seedlings grown on different concentrations of the chloro-anilines and chlorophenols.

Figure S2 Uptake and metabolism of [^{14}C]-DCA in reed canary grass and switchgrass over a 24-h exposure. Auto-radiograph of radioactive metabolites extracted from the grasses and resolved by thin layer chromatography with parent DCA and DCA-*N*-glucose identified.

Figure S3 Phytotoxicity of DCA in reed canary grass and switchgrass. Effect of exposing reed canary grass (Palaton) and switchgrass (Trailblazer) to 100 μM DCA for 12 days.

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